THE SOLUTION CONFORMATION OF THE PEPTIDE ANTIBIOTIC THIOSTREPTON: A ^{1}H NMR STUDY

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The majority of the 84 protons in the ^{1}H NMR spectrum of thiostrepton at 300 MHz were unambiguously assigned on the basis of double resonance experiments under different conditions of solvent, temperature and ^{2}H-exchange by comparison with the known crystal structure determined by ANDERSON *et al.*[1] Evidence is presented to suggest that the side chain, the nature of which remained undefined on X-ray analysis, is comprised of two dehydroalanine residues which supports the conclusions reached by TORI *et al.*[2] on the basis of ^{13}C NMR spectroscopy. These two residues are missing in thiostrepton A_2, a minor artifact. All available ^{1}H NMR evidence suggests thiostrepton to have a similar conformation in deuterochloroform solution to that found in the crystal form.

In our structural work on the thiopeptins[3], which is the subject of a companion paper, we became heavily dependent on the ^{1}H NMR assignments in the structurally related antibiotic thiostrepton, the structure of which was largely determined by X-ray crystallography by ANDERSON *et al.*[1]. Some doubt remained about the nature of the side chain which was subsequently shown to consist of two dehydroalanine (Deala) residues on the basis of ^{13}C NMR spectroscopy[2]. We have therefore undertaken an extensive ^{1}H NMR investigation of thiostrepton at 300 MHz under a wide variety of conditions which has resulted in unequivocal assignments of the majority of resonances. The ^{1}H NMR evidence was found to be consistent with its X-ray structure and unique conformation, as well as confirming the nature of the side chain. Some of our findings were reported independently by TORI *et al.*[4].

Experimental

A sample of thiostrepton was obtained from the Squibb Institute. On standing it slowly decomposed to a minor product of higher Rf on silica gel TLC and was designated thiostrepton A_2. It was easily separated from thiostrepton (for convenience assigned the A_1 component) by preparative TLC on silica gel GF (254) plates (Analtech) using CH_3OH - CH_2Cl_2 (1 : 19).

^{1}H NMR spectra of thiostreptons A_1 and A_2 were obtained using a Varian SC-300 MHz spectrometer in the Fourier Transform mode using acquisition times of 1 and 2 seconds with sweep width of 4,000 Hz. Concentrations were in the range 3.5 ~ 5.0 mg/0.4 ml and chemical shifts are quoted in δ, ppm downfield of TMS.

Crystal coordinates for two althiomycin derivatives were obtained from published data[5,6] from which torsional angles and internuclear bond distances were readily computed. Hydrogen positions in thiostrepton were calculated from the crystal coordinates[1] assuming standard distances and angles.

Results and Discussion

The structure and stereochemistry of thiostrepton A_1 as determined by ANDERSON *et al.*[1] is shown in Fig. 1 indicating the subunit designations and numbering system used. Protons above or below the plane of various ring systems are labelled β and α respectively, and the methylene protons of the Deala residues are designated *cis* and *trans* with respect to the peptide carbonyl group. The ^1H NMR assignments of thiostrepton A_1 and A_2 under various experimental conditions are summarized in Table 1 in increasing order of chemical shift. Spectra were measured in $CDCl_3$ and titrated with CD_3OD which resulted in immediate exchange of the hydroxylic protons in contrast to the slow disappearance of the peptide NH protons. The rate of exchange was sufficiently slow for all peptide NH signals to be recorded for $CDCl_3$ solution spiked with CD_3OD but not in CD_3OD - $CDCl_3$ (1: 4) (see columns 7 and 8, Table 1). Due to appreciable solvent shifts accompanying the addition of CD_3OD, excellent chemical shift dispersion was achieved allowing many assignments to be made from decoupling experiments. In particular double resonance experiments confirmed the assignments made on the basis of the multiplicity changes observed on ^2H-exchange as indicated in column 2 of Table 1.

We shall now consider in some detail specific assignments of the subunits of thiostrepton as shown in Fig. 1.

Fig. 1. Structure of thiostrepton.

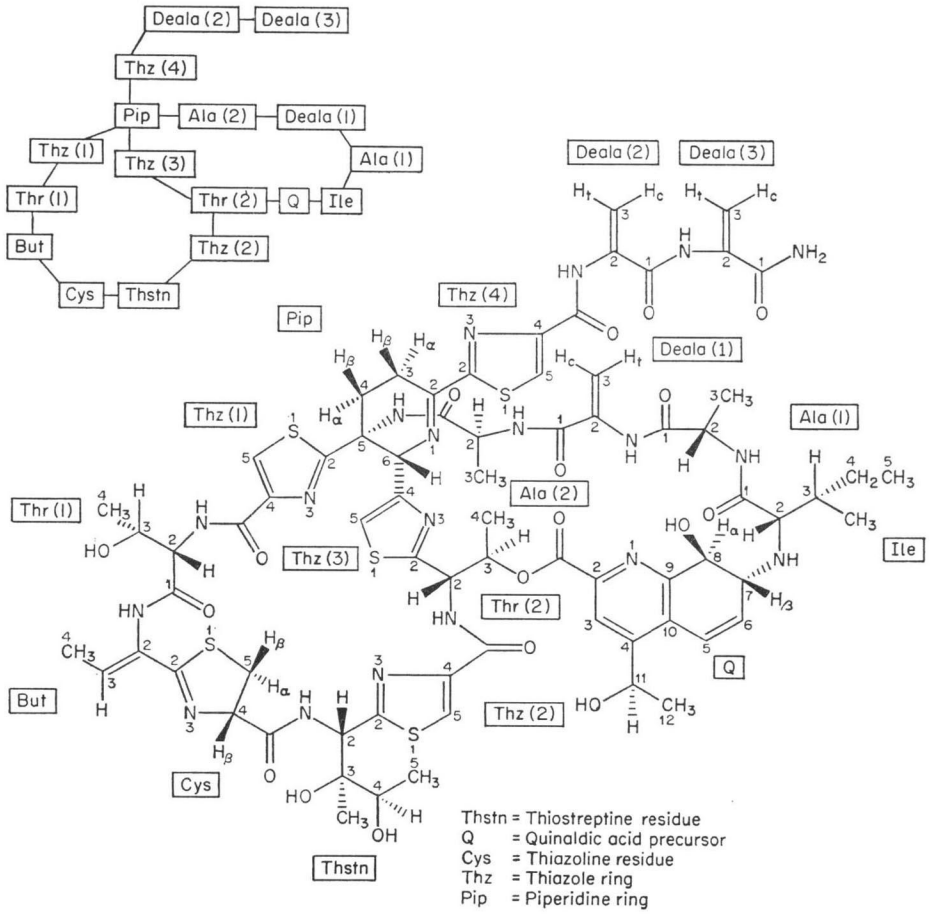


Table 1. ^{1}H NMR assignments of thiostrepton A_1 and A_2 in various solvent mixtures at 25 and 45°C.[a]

Assignment[b]	Effect of ^{2}H-exchange[c]	$CDCl_3$				$CDCl_3$(+CD_3OD)[d]	CD_3OD - $CDCl_3$ (1 : 4)[e]
		A_1 (25°C)	A_2 (25°C)	A_1 (45°C)	A_2 (45°C)	A_1 (25°C)	A_1 (25°C)
Ile 3-CH_3		0.88 d (7)	0.89 d (7)	0.90 d	0.90 d	0.92 d (7)	0.82 d (7)
Ile 5-CH_3		0.94 t (7)	0.96 t (7)	0.96 t	0.95 t	0.91 t (7)	0.90 t (7)
Thr CH_3(1)		0.98 d (7)	0.99 d (7)	1.01 d	1.00 d	0.99 d (7)	1.04 d (7)
Thr H-3(1)		1.06 m	1.06 m	obsc.	obsc.	obsc.	~1.63 m, obsc.
Ala CH_3(1)		1.17 d (7)	1.20 d (7)	1.19 d	1.19 d	1.20 d (7)	1.20 d (7)
Thstn 3-CH_3		1.19 s	1.19 s	1.19 s	1.19 s	1.16 s	1.18 s
Q CH_3		1.35 d (7)	1.34 d (7)	1.35 d	1.34 d	1.37 d (7)	1.42 d (7)
Thstn 5-CH_3		1.36 d (7)	1.37 d (7)	1.37 d	1.37 d	1.33 d (7)	1.32 d (7)
Ala CH_3(2)		1.46 d (7)	1.47 d (7)	1.48 d	1.46 d	1.47 d (7)	1.47 d (7)
But CH_3		1.62 d (7)	1.64 d (7)	1.65 d	1.65 d	1.62 d (7)	1.65 d (7)
Thr CH_3(2)		1.75 d (7)	1.76 d (7)	1.77 d	1.76 d	1.74 d (7)	1.76 d (7)
Ile H-3		~1.75 obsc.	~1.75 obsc.	obsc.	obsc.	1.84 m	1.98 m
Pip H-4β		2.28 dt (6, 12.5, 12.5)	2.28 t (6, 12.5, 12.5)	2.29 dt	2.28 dt	2.30 dt (6, 12.5, 12.5)	2.35 dt (6, 13, 13)
Pip H-3α		~3.00 obsc.	~3.00 obsc.	~3.00 obsc.	~3.00 obsc.	~3.00 obsc.	~3.00 obsc.
Ile H-2		3.01 d (6)	3.03 d (6)	3.03 d	3.02 d	3.00 d (6)	3.02 d (4)
Cys H-5α		3.15 dd (11.5, 13)	3.15 dd (11, 13)	3.16 dd	3.16 dd	3.16 dd (11, 13)	3.21 dd (11, 13)
Pip H-3β		3.50 dd (6, 19)	3.38 dd (6, 19)	3.60 dd	3.36 dd	3.52 dd (6, 19)	3.53 dd (6, 19)
Q H-7β		3.65 dd (1.5, 6)	3.65 dd (1.5, 6)	3.66 dd	3.65 dd	3.64 dd (1.5, 6)	3.67 dd (1.5, 6)
Cys H-5β		3.73 dd (9, 11.5)	3.74 dd (9, 11)	3.73 dd	3.72 dd	3.72 dd (9, 11)	3.69 dd (9, 11)
Thstn H-4	dq (5, 7) → q (7)	3.84 dq (5, 7)	3.84 dq (5, 7)	3.84 dq	3.84 dq	3.81 q (7)	3.85 q (7)
Ala H-2(1)	dq (6, 7) → q (7)	3.89 dq (6, 7)	3.88 dq (6, 7)	3.88 dq	3.89 dq	3.84 dq (6, 7)	3.85 q (7)
Thstn C4-OH	d (5) → o	4.09 d (5)	4.09 d (5)	3.99 br.s	3.96 br.s	—	—
Pip H-4α		~4.11 obsc.	~4.11 obsc.	4.14 dd (6, 12)	4.11 dd	4.12 dd (6, 12)	obsc. (HOD)[f]
OH/NH	br.s → o	4.12 br.s	4.12 br.s	~3.99 obsc.	~3.96 obsc.	—	—
Thr H-2(1)	dd (3, 8) → d (3)	4.48 dd (3, 8)	4.49 dd (3, 8)	4.49 dd	4.49 dd	4.49 dd (3, 8)	4.48 d (3)
Deala NH_2(3)[g]	br.s → o	4.54 br.s	7.22 br.s	~4.47 br.s	7.16 br.s	—	—

Table 1. (Continued)

Assignment[b]	Effect of ^{2}H-exchange[c]	$CDCl_3$				$CDCl_3$(+CD_3OD)[d]	CD_3OD - $CDCl_3$ (1 : 4)[e]
		A_1 (25°C)	A_2 (25°C)	A_1 (45°C)	A_2 (45°C)	A_1 (25°C)	A_1 (25°C)
Q H-8α	dd (1.5, 8) → d (1.5)	4.70 dd (1.5, 8)	4.71 dd (1.5, 8)	4.72 dd	4.72 dd	4.54 d (1.5)	4.43 d (1.5)
Ala H-2(2)	dq (7, 7) → q (7)	4.79 dq (7, 7)	4.80 dq (7, 7)	4.78 dq	4.78 dq	4.78 dq (7, 7)	4.78 q (7)
Cys H-4β		4.98 dd (9, 13)	4.99 dd (9, 13)	4.99 dd	4.99 dd	4.99 dd (9, 13)	5.02 dd (9, 13)
Deala H-3$_c$(1)	br.s → d (2.5)	5.12 br.s	5.13 br.s	5.12 br.s	5.11 br.s	5.28 br.s	5.38 d (2.5)
Pip H-6β		5.23 br.s	5.23 br.s	5.24 br.s	5.24 br.s	5.28 br.s	5.35 br.s
Q H-11		~5.34 q (7)	~5.36 q (7)	5.33 q	5.34 q	5.35 q (7)	~5.36 obsc.
OH/NH	s → o	5.36 s	5.37 s	5.30 br.s	5.30 br.s	—	—
Deala H-3$_c$(3)	br.s → d (2.5)	5.49 br.s	—	5.47 br.s	—	5.55 br.s	5.66 d (2.5)
Deala H-3$_c$(2)	br.s → d (2.5)	5.59 br.s	—	5.58 br.s	—	5.61 br.s	5.76 d (2.5)
'H_2O'	br.s → o	5.74 br.s (2H)[h]	~5.80 obsc.	—	5.69 br.s (2H)	—	—
Thstn H-2	d (10) → o	5.79 d (10)	5.80 d (~10)	5.81 d	5.82 d	5.77 d (10)	5.80 s
Deala H-3$_t$(1)		5.80 d (~2.5)	5.81 d (2.5)	5.81 d	5.82 d	5.84 d (2.5)	5.84 d (2.5)
Thr H-2(2)	d (10) → s	5.87 d (10)	5.88 d (9.5)	5.89 d	5.88 d	5.83 d (10)	5.84 s
But H-3	br.q (7) → q (7)	6.22 br.q (7)	6.23 br.q (7)	6.23 br.q	6.23 br.q	6.24 br.q (7)	6.28 q (7)
Q H-6		6.33 dd (6, 10)	6.35 dd (6, 10)	6.33 dd	6.34 dd	6.38 dd (6, 10)	6.46 dd (6, 10)
Ala NH(2)	d (7) → o	6.41 d (7)	6.39 d (7)	6.39 d	6.37 dd	6.85 d (7)	7.16 d → o
Thr H-3(2)		6.41 q (7)	6.41 q (7)	6.42 q	6.42 q	6.42 q (7)	6.41 q (7)
Deala H-3$_t$(3)		6.73 d (2.5)	—	6.72 d	—	6.69 d (2.5)	6.59 d (2.5)
Deala H-3$_t$(2)		6.83 d (2.5)	—	6.82 d	—	6.82 d (2.5)	6.77 d (2.5)
Q C8-OH	d (8) → o	6.86 d (8)	6.86 d (8)	6.74 br.s	6.72 br.s	—	—
Q H-5		6.92 d (10)	6.93 d (~10)	6.93 d	6.93 d	6.93 d (10)	6.95 d (10)
Thr NH(1)	d (8) → o	6.93 d (8)	6.93 d, obsc.	6.93 d	6.92 d	6.98 d (8)	7.16 d → o
Q H-3		7.34 s	7.35 s	7.34 s	7.34 s	7.34 s	7.38 s
Thz H-5(3)		7.49 s	7.50 s	7.50 s	7.49 s	7.52 s	7.63 s
Thstn C3-OH	s → o	7.54 s	7.51 s	7.36 br.s	7.26 br.s	—	—
Ala NH(1)	d (6) → o	7.60 d (6)	7.60 d (5.5)	7.56 d	7.55 d	7.73 d (6)	7.86 d → o
Thstn NH	d (10) → o	7.61 d (10)	7.61 d (10)	7.59 d	7.59 d	7.59 d (10)	7.63 d → o
Deala NH(1)	br.s → o	7.84 br.s	7.84 br.s	7.83 br.s	7.82 br.s	7.88 br.s	8.10 br.s → o

Table 1. (Continued)

Assignment[b]	Effect of ^{2}H-exchange[c]	$CDCl_3$				$CDCl_3$(+CD_3OD)[d]	CD_3OD - $CDCl_3$ (1:4)[e]
		A_1 (25°C)	A_2 (25°C)	A_1 (45°C)	A_2 (45°C)	A_1 (25°C)	A_1 (25°C)
Thz H-5(1)		8.15 s	8.15 s	8.16 s	8.16 s	8.18 s	8.23 s
Thz H-5(4)		8.30 s	8.30 s	8.30 s	8.28 s	8.32 s	8.35 s
Thz H-5(2)		8.31 s	8.31 s	8.30 s	8.27 s	8.33 s	8.36 s
Thr NH(2)	d (10) → o	8.32 d (10)	8.32 d (~10)	8.30 d	8.27 d	8.70 d (10)	8.87 d → o
But NH	br.s → o	8.55 br.s	8.56 br.s	8.54 br. s.	8.54 br.s	8.58 br.s	—
Deala NH(3)	br.s → o	9.03 br.s	—	9.00 br.s	—	9.06 br.s → o	—
Pip C5-NH	s → o	9.90 s	9.92 s	9.90 s	9.92 s	9.96 s	9.97 s → o
Deala NH(2)	br.s → o	10.00 br.s	—	9.98 br.s	—	10.03 br.s → o	—

[a] Chemical shifts in ppm downfield of internal TMS. Coupling constants in Hz (±0.5) are given in brackets.
Abbreviations: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, dt=doublet of triplets, q=quartet, dq=doublet of quartets, m=multiplet, br=broad, obsc.=obscured (overlapping signals).

[b] See Fig. 1 for subunits.

[c] Multiplicity changes on ^{2}H-exchange are indicated by →; complete disappearance of an active hydrogen is designated by s → o or d → o.

[d] Approximately CD_3OD - $CDCl_3$ (1:19).

[e] Ile 4-CH_2 was located at *ca* δ 1.20 by decoupling.

[f] At δ 4.13 dd (6, 13) at 45°C.

[g] The assignment is for Thz (4) $CONH_2$ in the case of A_2 at 65°C.

[h] At 65°C.

Assignment of Residues Containing Methyl Groups

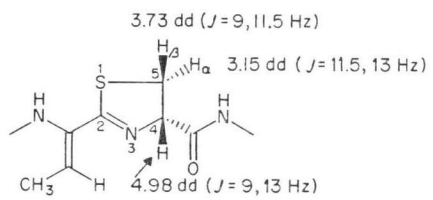
Thiostrepton A_1 has ten methyl groups which appear reasonably well separated as 1 singlet, 8 doublets, and 1 triplet. Systematic decoupling experiments in $CDCl_3$ have allowed assignment of the Ile, Thr (1) and two Ala resonances which were confirmed by peptide N*H* ^{2}H-exchange studies in CD_3OD - $CDCl_3$ (1 : 4). The almost 1.0 ppm difference in chemical shift between H2 of the two alanine residues can be used to assign them individually, by anticipating the X-ray conformation to be the same in solution. Ala H-2 (2) lies in the deshielding cone of the ester carbonyl of the dihydroquinoline ring (Q) and is thus assigned to the resonance at lower field. The anomalous chemical shift values of the Thr (1) residue will be discussed later. The two methylcarbinol resonances of the thiostreptine (Thstn) and dihydroquinoline (Q) moieties were readily assigned on the basis of chemical shift, leaving only the two most downfield methyl doublets at δ 1.62 and 1.75 to be assigned (see Table 1) to the butyrine (But) and modified Thr (2) residues. Assignment of the latter resonances was rendered difficult as H-3 appears as a quartet and H-2 as a doublet implying $J_{H2,H3} \simeq 0$ Hz. Consequently the pair of doublets for the CHNH protons at δ 5.87 and 8.32 (J=10 Hz) can not be readily distinguished from those for the Thstn residue which appear in the same part of the spectrum at δ 5.79 and 7.61 (d, J=10 Hz). Examination of the fully ^{2}H-exchanged spectrum, however, shows the two methine doublets at δ 5.79 and 5.87 as singlets with the latter of broader line-width. Irradiation of this broad singlet causes sharpening of the quartet at δ 6.41 (and *vice versa*) which in turn is coupled to the most downfield methyl group at δ 1.75. These resonances were therefore assigned to the Thr (2) residue and settle the question of assignment of the Thstn, Thr (2) and But residues.

Assignment and Conformation of Thiazoline Ring

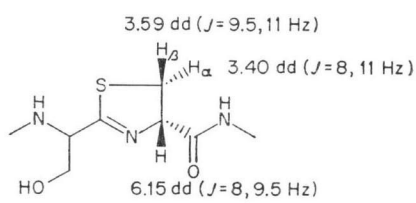
Successive irradiation of the three doublet of doublets at δ 3.15, 3.73 and 4.98 in thiostrepton A_1 resulted respectively in collapse of the other two resonances to doublets. The three protons thus form a first order AMX spin system and were assigned to the protons of the thiazoline ring. The configuration at C4 is R^1 as in althiomycin (**2**[6], Fig. 2), opposite in hand to the remaining amino acid residues. The assignment of H_α and H_β at C5 follows from the respective vicinal coupling constants of 9 and 13 Hz as discussed below.

The large vicinal coupling constant of 13 Hz for $J_{H4,H5}$ of the thiazoline ring in thiostrepton A_1 compared to 9.5 Hz observed for althiomycin (**2**[5], see Fig. 2) appears inconsistent but can be explained in conformational terms. The presence of a double bond in a five-membered ring eliminates the possibility of pseudo-rotation and thus reduces the number of possible conformations to a choice between a planar and envelope conformation[7]. There are many instances where such an envelope conformation

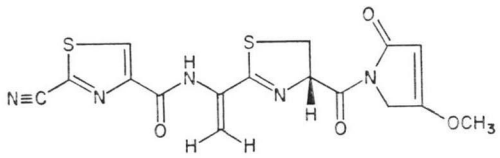
Fig. 2. Comparison of ^{1}H NMR assignments of thiazoline ring in thiostrepton A_1 (**1**) and althiomycin (**2**) in $CDCl_3$.



1 Thiostrepton (partial structure)



2 Althiomycin (partial structure)



3 Bis-anhydroalthiomycin

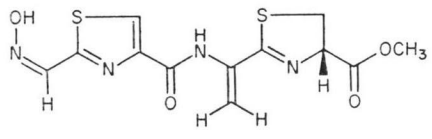


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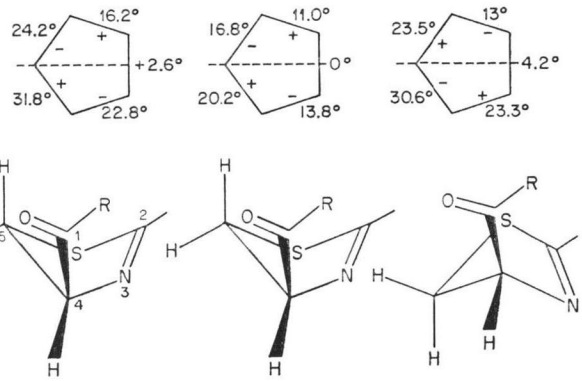
Fig. 3. Comparison of torsional angles, dihedral angles (θ) and conformations for thiazoline rings in **1**, **3** and **4** from X-ray data.



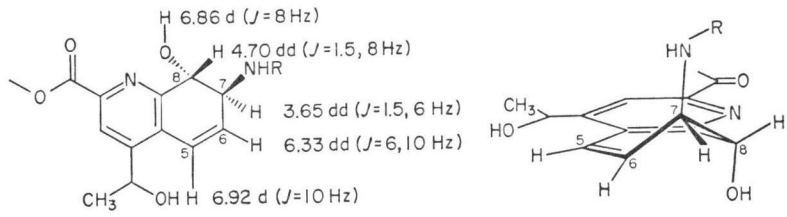
	1a (Thiostrepton)	**3a**	**4a**
$\theta_{cis}^{H4,H5}$	32° (J=9 Hz)	20° (J=9.5 Hz)	−30°
$\theta_{trans}^{H4,H5}$	152° (J=13 Hz)	140° (J=8 Hz)	−90°

is favored including that of the thiazoline ring found in the crystal structures of thiostrepton and two derivatives of anhydroalthiomycin **3** and **4**[5,6] as indicated by their torsional angles in Fig. 3. It is readily observed that the methyl ester **4** with the carboxymethyl group in the pseudo-axial orientation, has crystallized in the alternative envelope conformation to that in thiostrepton and bis-anhydroalthiomycin **3**. The values of 8 and 9.5 Hz for J_{vic} in althiomycin are strikingly similar to those observed for dihydrothiophene (7.5 and 10 Hz) and thiazoline (8.0 and 9.8 Hz)[5] and values comparable to those found in thiostrepton have only been observed in other five-membered heterocyclic ring systems such as pyrazolines[9] and isoxazolines[10] where increased substitution appears to lock the ring in a rigid conformation. The high value in thiostrepton can therefore be accounted for by retaining the frozen crystal conformation as depicted in **1a** (Fig. 3) in solution, with J_{trans}=13 Hz (θ=152°) and J_{cis}=9 Hz (θ=32°) making $J_{trans}>J_{cis}$. The values of 8.0 and 9.5 Hz for althiomycin reflect a conformational equilibrium between the two envelope forms **3a** and **4a** where now the usual relationship in five membered rings, $J_{cis}>J_{trans}$ holds. J_{cis} is almost identical in the equilibrating (**3a**) and non-equilibrating (**1a**) situations and in the absence of orientationally-dependent electronegativity effects, argues for similar dihedral angles in both envelope forms which is born out from the X-ray data.

Assignment and Conformation of Dihydroquinoline Ring

Proceeding in an analogous manner as for the thiazoline ring protons, double resonance experiments allowed the four contiguous methine protons of the dihydroquinoline moiety (Q) to be unequivocally assigned as shown in **5a** of Fig. 4. In addition to a small coupling to H-7, H-8 is coupled to the hydroxylic proton at C8 which is removed immediately on addition of a drop of CD_3OD (see Table 1). The

Fig. 4. ^{1}H NMR assignments ($CDCl_3$) and conformation (**5b**) of dihydroquinoline residue (Q) (**5a**).



5a **5b**

trans diaxial orientation of the substituents at C7 and C8 in the solid state as depicted in **5b** (Fig. 4), appears to be predominant in the solution structure where the dihedral angle between H-7 and H-8 is 72° in agreement with the small value of 1.5 Hz for $J_{H7,H8}$. Coupling between the diaxial protons in the alternative flattened half-chair conformation would predictably be large. Moreover the dihedral angle between H-6 and H-7 of 26° is consistent with a coupling constant of 6 Hz; also the absence of allylic coupling is consistent with the torsional angle of 26° defined by the planes containing C5-C6-C7 and C6-C7-H7.

Assignment and Conformation of Δ^1-Piperidine Ring

The chemical shifts of the CH_2CH_2 protons in the Δ^1-piperidine ring are anomalous due to the neighboring ring currents of the thiazole rings and analyze as a first order four spin system. Double resonance experiments have allowed the spin system to be characterized by the parameters summarized in Table 2. The geminal coupling constants of 12 and 19 Hz for $J_{4\alpha,4\beta}$ and $J_{3\alpha,3\beta}$ allow the allylic protons at C3 to be readily distinguished from those at C4. The dihedral angles between H-3β and H-4β and between H-3α and H-4α must of necessity be very similar which is borne out by $J_{3\beta,4\beta}=J_{3\alpha,4\alpha}=$ 6 Hz, suggesting a gauche relationship as depicted in the Newman projections for either half-chair conformation **a** or **b** in Fig. 5. The large J_{vic} of 12 Hz is in agreement with a predominantly *trans* diaxial relationship and the small J_{vic} of 1.0~1.5 Hz is consistent with a dihedral angle close to 90° which satisfies either conformation **a** or **b**. The broad singlet at δ 5.23 was assigned to Pip H-6 and shown by double resonance to be homoallylicly coupled to the C3 methylene protons. It is evident that the four vicinal coupling constants do not allow a distinction between the two half-chair conformations. However, a conformational dependence of homoallylic coupling constants has been demonstrated in similar half-chair conformations[11] where $J_{a'a'}>J_{a'e'}\simeq J_{e'a'}>J_{e'e'}$ as a consequence of the notably greater angle (ϕ) between the direction of the C-H bond and plane of the double bond associated with pseudoaxial configuration (a') than a pseudo-equatorial configuration (e'). Many examples have shown that for comparable geometry, replacement of carbon by sp^2-hydridized nitrogen does not result in gross changes in the magnitude of homoallylic constants[12] and on this basis, the large values of $J_{3\beta,6\beta}\simeq$ 1.5 Hz and $J_{3\alpha,6\beta}=3.5$ Hz therefore favor the half-chair conformation **a**.

Table 2. Vicinal and homoallylic coupling constants (Hz) in Δ^1-piperidine ring in thiostrepton A_1.[a]

	Pip H-3 (~3.00 m)	Pip H-3 (3.50 dd)	Pip H-4 (4.11 dd)	Pip H-4 (2.28 dt)	Pip H-6 (5.23 br. s)
H-3α	—	19	6	12	3.5
H-3α	19	—	1~1.5	6	1.5

[a] Chemical shifts in $CDCl_3$ (δ ppm) are given in brackets.

Fig. 5. Half chair conformations and Newman projections about C4–C3 bond of Δ^1-piperidine ring in thiostrepton.

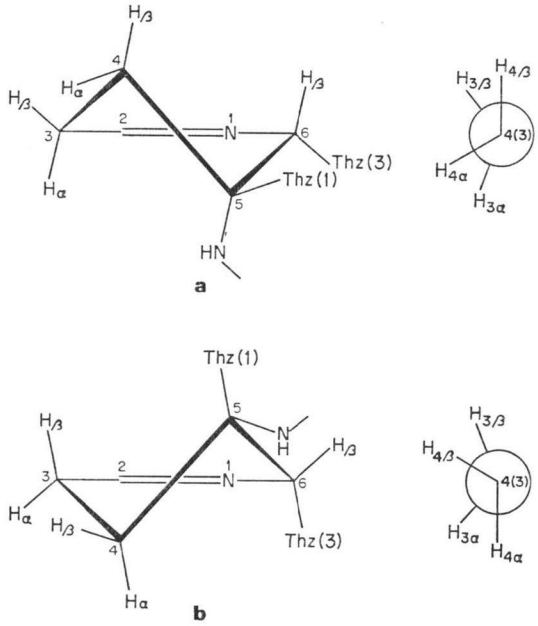
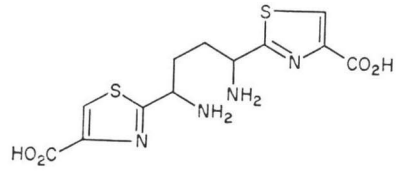


Fig. 6. Comparison of torsional angles in cyclohexene with that of Δ^1-piperidine ring in thiostrepton.

Comparison of the torsional angles for the Δ^1-piperidine ring calculated from the X-ray data for thiostrepton, with that for cyclohexene[13] in Fig. 6, indicates that both rings assume the half-chair conformation **a** (Fig. 5). Calculation of the four dihedral angles between the four CH_2CH_2 protons *i.e.* 47°, 47°, 73° and 167° shows them to be consistent with the above analysis from vicinal constants. Furthermore, the angles of 80°, 72° and 48° which the bonds C-$H_{\epsilon\beta}$, C-$H_{\delta\alpha}$ and C-$H_{\epsilon\beta}$ make with the plane of the imine double bond respectively, are fully consistent with the $\sin^2\phi$ $\sin^2\phi'$, dependence[12] of the homoallylic coupling constants anticipated for conformation **a**. This same conformation as in the crystal structure of thiostrepton adequately accounts for the deshielding of H-3α and H-3β by the thiazole ring Thz (4), as well as H-4α and Pip C5-NH proton by Thz (1), both of which occur considerably downfield at δ 4.11 and 9.90 respectively. Moreover H-6β falls in the deshielding zone of both Thz (1) and Thz (3) and is found, shifted downfield to δ 5.23. The large J_{gem} of 19 Hz for the allylic protons at C3 is consistent with conformation **a** (Fig. 5) in which the plane of the imine double bond almost bisects the H–C3–H angle[14]. This also holds for conformation **b** and therefore does not by itself allow a distinction between the two forms to be made.

The above assignments were confirmed by examining thiostrepton A_1 in CD_3OD - $CDCl_3$ (1 : 4) spiked with trifluoroacetic acid-*d* (TFA-*d*). It was observed that resonances H-3α and H-3β gradually disappear while H-4α and H-4β simplify to doublets (J=12 Hz). Moreover the homoallylic couplings to H-6β are now removed and H-6β appears as a clean sharp singlet. No change in the chemical shifts of H-4α and H-4β and in particular H-6β were noted and is interpreted in terms of an imine-enamine equilibrium, lying almost completely to the unprotonated imine side. Partial exchange was someobserved intimes CD_3OD - $CDCl_3$ (1 : 4) in the absence of TFA-*d*.

It should be mentioned that the position of the double bond in the piperidine ring of the crystal structure of thiostrepton as determined by ANDERSON and coworkers[1] presented some initial difficulty. Acid hydrolysis was shown to lead to thiostreptoic acid (**6**) whereas 2-(1-amino-2-carboxyethyl)thiazole-4-carboxylic acid (**7**) was the product if preceded by an oxidation step with peracid[15]. Neither of these compounds is an obvious hydrolysis product which suggested to the above authors that the double bond must shift between N1 and C6 to allow the piperidine ring to be degraded in two different ways. A double bond shift of the imine-enamine type as suggested by the ^{1}H NMR experiment with TFA-*d* is not unusual



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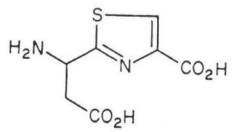

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but one across nitrogen under acidic conditions cannot be readily explained. The ^{1}H NMR results nevertheless locate the position of the double bond conclusively between N1 and C2 and not N1 and C6 in support of the conclusions reached by ANDERSON *et al.*[1] from a difference map.

Nature of Side Chain and Assignment of Exchangeable Protons

The temperature dependence of the active OH and one NH protons in $CDCl_3$ and their subsequent fast rate of exchange with CD_3OD allows them to be readily distinguished from the peptide NH protons. With increasing temperature, six resonances move upfield with appreciable broadening whereas all peptide NH protons move only slightly upfield and remain as sharp singlets or doublets. Subsequent analysis of the CD_3OD spiked spectrum confirmed their presence in apparent agreement with the number of one basic Ile NH and five OH groups (but see below). Their presence was moreover confirmed by a saturation transfer experiment[16] which involved irradiation of the "bulk" water peak near δ 2.70 with which the protons are in fast exchange. All six resonances are effectively reduced in intensity under these conditions whereas the peptide NH resonances are unaffected. By contrast this technique has in the past been used for the detection of peptide NH resonances in aqueous solution and only recently in a non-aqueous solvent (DMSO-d_6) containing a small amount of water[17].

Under complete ^{2}H-exchange, the ^{1}H NMR spectrum shows five aromatic singlets at δ 7.38, 7.63, 8.23, 8.35 and 8.36 which are attributed to H-3 of the dihydroquinoline moiety and four thiazole H-5 protons. These were individually assigned by comparison with thiopeptin A_{1a} and A_{1b} (see companion paper). This leaves only 6 doublets (J=2.5 Hz) unassigned. A pairwise connection between these doublets was established on the basis of decoupling experiments, highly characteristic of the presence of three Deala residues[15], one in the ring and two in the side chain. The latter resonances are conspicuously missing in the spectrum of thiostrepton A_2. Re-examination of the spectrum of thiostrepton A_1 in $CDCl_3$ (see Table 1) revealed the upfield member of each pair as a broad singlet instead of a clean doublet as observed for the other member of the pair which suggested stereospecific allylic coupling to the respective NH protons. In fact, irradiation of the peptide NH singlets at δ 7.84, 9.03 and 10.00 in $CDCl_3$ resulted in sharpening of the broad singlets at δ 5.12, 5.49 and 5.59 to doublets (J= 2.5 Hz) which therefore unambiguously assigns each of the three peptide NH protons to its respective Deala residue. Assuming the allylic coupling to be of the transoid type, the proton *cis* to the peptide carbonyl group can be assigned to the resonance at higher field. This assumption appears justified as a similar coupling was observed between the transoid arrangement of But H-3 at δ 6.22 and But NH at δ 8.55. The remaining NH singlet at δ 9.90 is therefore assigned to Pip C5-NH. By comparison with thiostrepton A_2 and thiopeptins $A_{3a/3b}$ and $A_{4a/4b}$ (see companion paper) which have the terminal Deala residues missing, unequivocal assignment of resonances for each residue was possible.

The ^{1}H NMR results are therefore consistent with the X-ray structure of thiostrepton A_1 and support the conclusions reached by TORI *et al.*[2,4] that the side chain consists of two Deala residues. By analogy with thiopeptin $A_{3a/3b}$, bis-de-Deala degradation products of thiopeptin $B_{a/b}$, the artifact

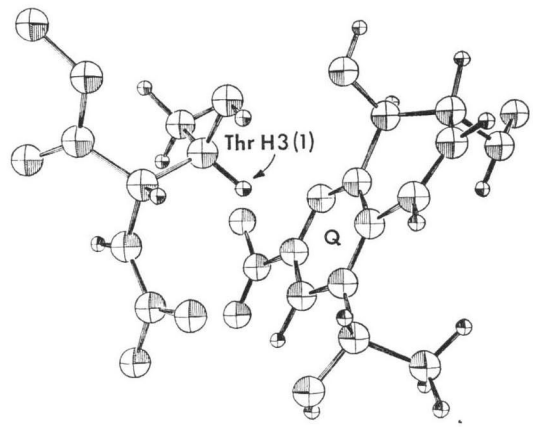
thiostrepton A_2 is assigned the bis-de-Deala derivative of A_1*.

Conformation of Thiostrepton A_1

In the foregoing discussion, the existence of a unique conformation for the thiazoline, Δ^1-piperidine and dihydroquinoline ring system has been proposed similar to those in the crystal conformation of thiostrepton which, as separate entities, might otherwise be expected to exhibit dynamic conformational equilibria. This lends support to the idea that the molecule overall retains a high degree of conformational rigidity in solution and it will be further demonstrated that other conformational features in solution can be accommodated by the crystal structure conformation.

Of significant interest is the observation that H-3 of the Thr (1) residue, which occurs at δ 3.93 in the threonine peptide CF_3CO-Gly-Gly-Thr-L-Ala-OCH_3[20] can be located by spin decoupling in the methyl group region at 1.06, shifted to higher field by almost 3.0 ppm. The reason for this dramatic upfield shift is amply evident from the crystal structure conformation (see ORTEP drawing[21] in Fig. 7) where the proton in question is situated directly over the center of the pyridine ring of the dihydroquinoline moiety (Q) and is thus exposed to the shielding effects of the aromatic nucleus. The actual distance from H-3 to the center of the pyridine ring is only 2.36 Å with $\theta = 86°$ where θ is the angle between the vector joining H-3 to the center of the ring with the plane of the ring. From the Johnson-Bovey Tables,[22] giving shieldings for a proton placed in or out of the plane of a benzene ring, the predicted shielding is 3.25 ppm, in close agreement with our observed value.

Fig. 7. Relationship of Thr H-3 to dihydroquinoline ring in thiostrepton (ORTEP drawing).



The crystal conformation[1] is maintained by a number of intramolecular hydrogen bonds which bind the whole molecule into a very compact, roughly globular structure with the two large rings folded over one another and the side chain protruding from the junction at the Pip nucleus. Hydrogen bonding reduces the rate of exchange of OH protons, which often allows them to be observed by ^{1}H NMR as sharp resonances and moreover are often found to lower field from their normal positions. One of these hydrogen bonds involves the hydroxyl groups of residues Thr (1) and Q at C8, where the latter OH proton has already been rigorously assigned by double resonance to the sharp doublet at δ 6.86 (J_{H8-OH} = 8.0 Hz). This hydrogen bond, as well as the van der Waals contacts at the L-threonine level similar to that found in the X-ray structure of the related antibiotic nosiheptide[23], as manifested in the large upfield shift of Thr H-3 (1), add considerable stability to this conformation.

The 'linchpin' of the thiostrepton conformation was described[1] as an oxygen atom, presumed to belong to a water molecule, which is placed within hydrogen bonding distance of the dihydroquinoline ring nitrogen, the peptide nitrogen of Thr (2) and the hydroxyl oxygen at C3 of the thiostreptine (Thstn) residue. We believe this molecule of water to be present in the solution conformation in $CDCl_3$ and in slow exchange with 'bulk' water, on the basis of the following analysis. We have seen above that at 25°C, thiostrepton A_1 as well as A_2 have six OH/NH resonances. One of the six signals, which appears

* Thiostrepton A_2 appears to be identical to thiostrepton B in reference 19.

at δ 4.54 in A_1 and δ 7.22 in A_2, integrates for *ca* two protons and strongly suggests observation of the Deala (3) $CONH_2$ and Thz (4) CONH protons respectively. Furthermore A_2 displays an additional two proton resonance at δ 5.69 at 45°C which can be clearly seen at δ 5.74 in A_1 at 65°C which we attribute to a slowly exchanging water molecule. Confirmation comes from the spectrum of thiopeptin A_{3a} (see companion paper). Because the molecule lacks the two Deala residues of the side chain as in thiostrepton A_2, it has the water and amide protons at similar positions at δ 5.69 and δ 7.17 respectively. To obtain the correct active hydrogen count, it must be assumed that either the Ile NH or one of the five OH protons is exchange broadened. We know from our studies on the thiopeptins (see companion paper) that the piperidine NH, characteristic of only the **a** series, is not observed and neither is it coupled to protons on the adjacent carbons because of an unfavorable exchange rate. It is therefore reasonable to assume that the basic Ile NH proton, not coupled to either Ile H-2 or Q H-7β in both thiostrepton A_1 and A_2, is also exchange broadened. Indirect evidence for the presence of this molecule of water and its role as the 'linchpin' through hydrogen bonding in the thiostrepton conformation, comes from the following observations: a) Thstn C3-OH is the only tertiary alcohol group in thiostrepton and is therefore expected to be a sharp singlet if not exchange broadened. This proton is assigned to the singlet at δ 7.54 and its extremely sharp nature and occurrence to low field is consistent with its participation in a strong hydrogen bond. b) With the exception of the Pip C5-NH and dehydropeptide NH protons, the peptide NH resonance found to lowest field is that for Thr NH (2) at δ 8.32 which is also one of the slowest to exchange (see below). c) Addition of CD_3OD results in a conformational change details of which are not certain but strongly suggesting unfolding of the 'wings' of the structure held together by the water 'linchpin' on replacement by methanol. This is primarily reflected in the shifts of some of the resonances for the dihydroquinoline moiety (Q), Thr (1) and Ile residues.

Backbone Conformation

Estimation of dihedral angles (θ) from coupling constants for peptide H–C_α–N–H fragments were made using BYSTROV *et al.*'s[24] refinement of the Karplus equations. In general there are four permissible angles for a given coupling constant as shown in Table 3 for the respective peptide fragments in thiostrepton. Where a large coupling constant is observed, the orientation is unambiguously *trans* as observed for the Thstn and Thr (2) fragments in agreement with that found in the crystal structure. In the other three cases, the possibility of averaging between different conformations is not ruled out, but the fact that one of the calculated dihedral angles is found to be in good agreement with that observed in the crystal structure may be taken as support for a relatively rigid backbone conformation of these resi-

Table 3. Comparison of solution (θ_{soln}) and crystal (θ_{xtal}) backbone torsional angels of peptide residues on thiostrepton[a].

Residue	J_{CHNH} (Hz)	θ_{soln}[b]	θ_{xtal}[c]	$180\pm\omega_{obs}$[c]
Thr (1)	8.0	$\pm$16 or $\pm$147°	+158°	−2.1°
Thr (2)	10.0	$\pm$162°	−156°	6.2°
Thstn	10.0	$\pm$34 or $\pm$136°	−152°	0.2°
Ala (1)	6.0	$\pm$34 or $\pm$136°	+129°	−1.4°
Ala (2)	7.0	$\pm$26 or $\pm$141°	+165°	−18.2°

[a] $\theta=\phi\pm60°$ where θ is the dihedral angle between the H–N–C_α and N–C_α–H planes; ϕ and ω as defined in reference 25.

[b] Calculated from BYSTROV *et al.*'s[24] relationship between J_{CHNH} and θ (see text).

[c] Calculated from crystallographic data.

dues. The value of J_{CHNH}=7 Hz for Ala (2) is lower than that expected for θ=165° observed in the crystal, but may be a consequence of the nonplanarity of the amide group ($180 \pm \omega = -18°$) in contrast to the near planarity of all other amide groups (see Table 3).

Side Chain Conformation

Side chain dihedral angles (χ) for the H-C_α-C_β-H fragment in Thr (1), Thr (2) and Ile were calculated using KOPPLE's equation[26] and are compared in Table 4 with those observed in the crystal structure. The possibility of averaging cannot be ruled out for the Ile residue but the good agreement, between observed and one of the calculated values for the threonine residues, and the fact that the couplings are both small, argues for fixed rotamers about these bonds similar to those found in the crystal form. This is not unexpected from the foregoing discussion on their involvement in intramolecular hydrogen bonding.

Table 4. Comparison of solution (χ_{soln}) and crystal (χ_{xtal}) side chain torsional angles of peptide residues in thiostrepton.

Residue	$J_{\alpha\beta}$ (Hz)	χ_{soln}[a]	χ_{xtal}[b]
Thr (1)	3.0	±62 or ±109°	+72°
Thr (2)	1.5	±81 or ±90°	−70°
Ile[c]	6.0	±40 or 128°	−60°

[a] Defined in reference 25 as the dihedral angle between the planes H-C_α-C_β and C_α-C_β-H. Calculated from KOPPLE's[26] relationship between $J_{\alpha\beta}$ and χ (see text).
[b] Calculated from crystallographic data.
[c] Ile is not strictly a peptide residue.

Hydrogen Bonding Involving Peptide CONH Protons

Various ^{1}H NMR methods have been employed to detect intramolecular hydrogen bonds involving the peptide protons[27]. One such method involves the rate of exchange of the CONH protons which were determined in CD_3OD - $CDCl_3$ (1: 19 and 1: 9) eventhough complete exchange of some of the resonances under these conditions was not realized within one week at ambient temperatures. The sequence, in order of decreasing rate was found to be: Deala (3)>Deala (2)>Thstn>Ala (2)>But>Pip C5≃Deala (1)>Thr (2)≃Thr (1)≃Ala (1). This order, preserved in column 1 of Table 5, is compared with the magnitude of the induced shift in the presence of CD_3OD and temperature coefficients in $CDCl_3$ and CD_3OD - $CDCl_3$ (1: 4) between 25 and 65°C. No simple correlation exists between the data obtained by the three methods which is attributed to conformationally induced changes in resonance

Table 5. Comparison of solvent shifts and approximate temperature coefficients for peptide CONH protons in thiostrepton.

Residue	δ $CDCl_3$	$\Delta\delta$[a]	$\Delta\delta$[b]	$\times 10^{-3}$ ppm/°C[c]	$\times 10^{-3}$ ppm/°C[d]
Deala (3)	9.03	−0.03	[e]	+1.8	[e]
Deala (2)	10.00	−0.03	[e]	+2.0	[e]
Thstn	7.61	+0.02	−0.02	+1.0	+0.5
Ala (2)	6.41	−0.44	−0.75	+1.3	+5.8
But	8.55	−0.03	[e]	+1.3	[e]
Pip C5	9.90	−0.06	−0.07	+0.5	+1.3
Deala (1)	7.84	−0.04	−0.26	+1.0	+4.0
Thr (1)	6.93	−0.05	−0.23	0	+1.0
Thr (2)	8.32	−0.38	−0.55	+2.3	+4.3
Ala (1)	7.60	−0.13	−0.26	+2.3	+3.8

[a] $\Delta\delta$=Shift (ppm) on spiking with CD_3OD.
[b] $\Delta\delta$=Shift (ppm) on addition of 20% CD_3OD. A negative sign indicates a downfield shift.
[c] Temperature coefficients in $CDCl_3$.
[d] Temperature coefficients in CD_3OD - $CDCl_3$ (1: 4).
[e] No data because of very fast exchange.

positions associated with the addition of CD_3OD. These are particularly marked for those resonances close to the thiazole and dihydroquinoline ring currents (see Table 1). As anticipated, the side chain Deala NH protons are first to exchange, followed by those of Thstn, Ala (2) and But on the surface of the molecule and not involved in hydrogen bonding. Of the slowly exchanging CONH protons, Thr NH (2) is hydrogen bonded and in addition to Thr NH (1) and Ala NH (1) is situated in a more hydrophobic environment. Moreover the Ala NH (1) proton and Ile nitrogen atom are within hydrogen bonding distance of each other, which, in addition to the amine being sterically crowded, may account for its difficulty to protonate with TFA-*d**. The crystal structure conformation therefore provides a reasonable rationale for the exchange rates of the majority of peptide CONH protons as well as the foregoing interpretation of the chemical shift and coupling constant data obtained in deuterochloroform solution.

Acknowledgments

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* No shifts of the protons adjacent to the Ile NH group, *i.e.* Q H-7 and Ile H-2 were observed in CD_3OD - $CDCl_3$ (1: 4) containing 5% TFA-*d*, under conditions where the allylic protons at Pip C3 readily exchange.

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